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provide valuable insights into its mode of action. The molecular framework of DFBP resembles that of levosimendan, thus it was chosen to mimic levosimendan to establish how the cTnC-cTnI binding equilibrium is modulated. We have utilized 2D {1H, 15N} HSQC and 2D {1H, 13C} HSQC NMR spectroscopy to examine the binding of DFBP to cNTnC•Ca2+ in the absence and presence of cTnI144-163 and of cTnI144-163 to cNTnC•Ca2+ in the absence and presence of DFBP. The results show that DFBP and cTnI144-163 bind cNTnC•Ca2+ concurrently and the affinity of DFBP for cNTnC•Ca2+ is increased ~5-10-fold by cTnI144-163. We are in the process of determining the NMR solution structure of cNTnC•Ca2+•cTnI144-163•DFBP. This structure will contribute to the understanding of the mechanism of action of levosimendan in the therapy of heart disease. It will also provide a structural basis for the design of Ca2+-sensitizing drugs in general.

1192-Pos Board B36

Decreased Fatigue Tolerance In Diaphragm Muscle Of Slow Troponin T Knockdown Mice

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The loss of slow skeletal muscle troponin T (TnT) results in a severe type of nemaline myopathy in the Amish (ANM). The genes encoding TnT and troponin I (TnI) are closely linked in pairs in which the 5′-enhancer region of the slow TnT gene overlaps with the cardiac TnI gene. In a mouse line with the entire cardiac TnI gene deleted, a partial destruction of the slow TnT gene promoter produces a knockdown effect. By crossing with transgenic mouse lines that over-express a core structure of cardiac TnI (cTnI-ND) under the control of cloned alpha-MHC promoter, we rescued the postnatal lethality of the cardiac TnI gene-deleted mice with no detrimental cardiac phenotypes or leaking expression in non-cardiac tissues. The double transgenic mice exhibited decreased expression of slow TnT mRNA and protein in adult diaphragm muscle. Functional analysis of isolated muscle strips showed that the slow TnT deficient (sTnT-KD) diaphragm had significantly decreased fatigue tolerance evident by the faster decrease in force and slower rate of recovery as compared with that in wild type controls. As a consequence of slow TnT deficiency, the sTnT-KD diaphragm muscle contained a higher proportion of fast TnT, decreased slow TnI with increased fast TnI, and decreased type I myosin with increased type II myosin. Consistent with the switch toward fast myofilament contents, the sTnT-KD diaphragm muscle produced higher specific tension in twitch and tetanic contractions as well as shorter time to develop peak tension in twitch contractions. The decreased fatigue tolerance of sTnT-KD diaphragm muscle explains the terminal respiratory failure seen in virtually all ANM patients and this double transgenic mouse model provides a useful experimental system to study the pathogenesis and treatment of ANM.

1193-Pos Board B37

Troponin Isoforms and Stretch-activation of Insect Flight Muscle

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Oscillatory contraction of insect indirect flight muscle (IFM) is activated by sinusoidal length changes. Work done by oscillating fibres is measured from the area of loops on a length-tension plot. At [Ca2+] above 10 μM, progressively less oscillatory work is produced because fibres contract isometrically and are unable to relax fully after each cycle of oscillation. Periodic stretches during oscillations activate fibres through the action of TnC F1, which binds one Ca2+ in the C-lobe. Activation of isometric contraction by Ca2+ acts through F2, which binds Ca2+ in both N- and C-lobes. Lethocerus IFM fibres substituted with F1gave oscillatory work, which did not decline at high [Ca2+], while fibres substituted with F2 produced more isometric tension as [Ca2+] was increased. Varying proportions of F1 and F2 gave maximal work with an F1:F2 ratio of 100:1, which is higher than the in vivo ratio of 7:1. The structure of F1, and the interaction with TnI, were determined by NMR. The N-lobe of F1 is in the closed conformation in apo and Ca2+- bound forms and does not bind TnI. Unexpectedly, the C-lobe is open in both states, and binds the N-terminal domain of TnI independently of Ca2+. The affinity of F1and F2 for a complex containing tropomyosin, TnT and TnH (Lethocerus TnI) were measured by isothermal calorimetry in the presence of Ca2+. The affinities of F1 and F2 for the complex were 5.4 μm and 65 nM respectively. This difference is likely to be due to a single TnI binding site on F1 and two sites on F2. Stretch may be sensed by an extended C-terminal domain of TnH, and transmitted to the C-lobe of F1, resulting in a change in the interaction of the TnI inhibitory domain and actin.

1194-Pos Board B38

Tracking of Qdot Conjugated Titin Antibodies in Single Myofibril Stretch Experiments Reveals Ig-domain Unfolding at Physiological Sarcomere Lengths

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The mechanical characteristics of titin in muscle sarcomeres were previously studied by us in single myofibril stretch experiments, where the extensibility of I-band titin segments was usually measured under static conditions. Here we investigated the behavior of I-band titin during and after stretch of single rabbit psoas myofibrils in real-time. The focus was on titin's proximal Ig-domain region, whose stretch dynamics were analyzed by labeling the myofibrils specifically in the N2A-titin domain using antibody-conjugated quantum dots, which stained the periphery of the myofibril but did not enter the myofilament lattice. Qdot labels were tracked to obtain the stretch-dependent change in epitope distance (across Z-disc) and sarcomere length (SL) over time. In contrast to what was expected from the current titin extensibility model, at sarcomere lengths of 2.5 and 3.8 μm, titin's proximal Ig-domain region elongated continuously, in proportion to the half I-band length. Already at ~2.6 μm SL the proximal Ig-segment length exceeded the value expected if all Ig-domains remain folded. Our results suggest that Ig-domains unfold in parallel with PEVK-titin extension at physiological sarcomere lengths and under relatively low forces. By reducing the antibody-Qdot concentration, we succeeded in observing titin Ig-domain dynamics in myofibrils at the single-molecule level.

1195-Pos Board B39

Constitutive Phosphorylation of Cardiac Myosin Binding Protein-C Increases the Probability of Myosin Cross-bridge Interaction with Actin

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Protein kinase A-mediated (PKA) phosphorylation of cardiac myosin binding protein-C (cMyBP-C) accelerates the kinetics of cross-bridge cycling and appears to relieve the tether-like constraint of myosin heads imposed by cMyBP-C (Colson *et al.*, 2008, *Circ Res.*, 103:244-251). We favor a mechanism in which phosphorylation of the 3 PKA sites in cMyBP-C modulates cross-bridge kinetics by regulating the proximity and interaction of myosin with actin. To test this idea, we used synchrotron low-angle x-ray diffraction and mechanical measurements in skinned myocardium isolated from a mouse model with phosphomimetic substitutions in cMyBP-C, i.e., the CTSD mouse. The substitutions were introduced by transgenic expression of cMyBP-C with Ser-to-Asp mutations on a cMyBP-C null background. Western blots showed that expression of CTSD cMyBP-C was 85% of wild-type (WT), and the heart weight to body weight ratio was similar (5.2 ± 0.2 mg/g) in CTSD and WT mice. Expression of WT cMyBP-C on the knockout background served as control (i.e., the CTWT mouse). Skinned myocardium from CTSD and CTWT mice exhibited similar maximum active forces (mN/mm^2: 17.7 ± 3.7 *vs* 13.2 ± 2.9), Ca^{2+}-sensitivities of force (pCa$_{50}$: 5.55 ± 0.03 *vs* 5.58 ± 0.04), and maximum rates of force development (k_{tr}, sec^{-1}: 20.2 ± 1.7 *vs* 22.5 ± 1.9; k_{df}, sec^{-1}: 37.6 ± 3.7 *vs* 43.2 ± 2.3). I_{11}/I_{10} intensity ratios and d_{10} lattice spacings determined from equatorial reflections from CTSD and CTWT myocardium were used to determine the effect of constitutive cMyBP-C phosphorylation on the distribution of cross-bridge mass between the thick and thin filaments and on interfilament lattice spacing. The results suggest that interactions between cMyBP-C and the S2 domain of myosin heavy chain are dynamically regulated by phosphorylations in the cMyBP-C motif. *(AHA-predoctoral fellowship (BAC); NIH-HL-R01-82900)*

1196-Pos Board B40

Obscurin Interacts with a Novel Isoform of Myosin Binding Protein C-Slow to Regulate the Assembly of Thick Filaments

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Obscurin is a multidomain protein composed of adhesion and signaling domains that plays key roles in the organization of contractile and membrane structures in striated muscles. We used adenoviral-mediated gene transfer to overexpress its extreme NH2-terminus in developing myofibers, followed by immunofluorescence and ultrastructural methods to study its effects in sarcomerogenesis. We found that overexpression of obscurin's second immunoglobulin domain (Ig2) inhibits the assembly of A- and M-bands, but not Z-disks and

I-bands. This effect is mediated by the direct interaction of obscurin's Ig2 with a novel isoform of the thick filament associated protein, Myosin Binding Protein-C Slow (MyBP-C slow), that corresponds to transcript variant-1. Variant-1 contains all the structural motifs known to be present in MyBP-C slow (variant-3), but has a unique COOH-terminus consisting of twenty-six amino acids and a new termination codon. RT-PCR showed that variant-1 is abundantly expressed in skeletal muscles during development and at maturity. Quantitative RT-PCR further demonstrated that transcripts containing the novel COOH-terminus are expressed in higher amounts than those lacking it. Three different antibodies to the unique COOH-terminus of variant-1 labeled M-bands and flanking regions in both developing and adult myofibers, suggesting that unlike other forms of MyBP-C that reside in C-zones, variant-1 preferentially concentrates in the middle of the A band. Adenoviral overexpression of obscurin's Ig2 domain and reduction of obscurin via siRNA inhibited the integration of variant 1 of MyBP-C slow into forming M-bands in skeletal myotubes. Collectively, our experiments identify a new ligand of obscurin at the M-band, MyBP-C slow variant-1, and suggest that its interaction with obscurin contributes to the assembly and maintenance of M- and A-bands.

Excitation - Contraction Coupling: Skeletal

1197-Pos Board B41

Functional Identification Of Fiber Types In Enzymatically Dissociated Murine Flexor Digitorum Brevis (FDB) And Soleus Muscles

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Enzymatically dissociated FDB and soleus fibers from mouse were used to compare the kinetics of electrically elicited Ca^{2+} transients of slow and fast skeletal muscle fibers, using the fast Ca^{2+} dye MagFluo4-AM, at 20-22°C. For the case of FDB fibers we found two different morphologies for both single-twitch and tetanic Ca^{2+} transients named morphology type I (MT-I) and morphology type II (MT-II). The kinetic parameters (mean ± s.e.m) of MT-I (11 fibers, 19%) and MT-II (47 fibers, 81%) single-twitch transients were: amplitude (ΔF/F): 0.36 ± 0.03 vs. 0.69 ± 0.03; rise time (ms): 1.44 ± 0.15 vs. 1.01 ± 0.02; half-amplitude width (ms): 10.25 ± 0.92 vs. 3.87 ± 0.12; decay time (ms): 46.15 ± 1.99 vs. 21.08 ± 0.89; and time constants of decay (τ_1 and τ_2, ms): 2.57 ± 0.19 and 33.71 ± 2.29 vs. 1.51 ± 0.05 and 13.19 ± 0.63, respectively; all differences being statistically significant ($p<0.001$). All Ca^{2+} transients parameters of soleus fibers ($n=20$) were not different ($p>0.1$) from those of MT-I FDB fibers. Tetanic responses (100 Hz) of MT-I FDB and soleus fibers showed a staircase shape while the time course of decay followed a single exponential (τ, ms): 73.36 ± 6.82 for FDB and 74.59 ± 6.24 for soleus (both $n=8$). In MT-II FDB tetani no staircase was present, the first peak was larger than the others, and the time course of decay was bi-exponential (Capote et al, J Physiol 2005;564:451). Histochemical and biochemical characterization of both muscles suggest that signals assigned MT-I correspond to slow type I and fast IIA fibers while those assigned MT-II correspond to fast IIX/D fibers. The results point to the importance of Ca^{2+} signaling for characterization of muscle fibers, but also to its possible role in determining fiber function. (FONACIT G-2001000637 and G-2005000372). JCC was supported by Universidad de Antioquia, Colombia.

1198-Pos Board B42

Contribution Of Ryr1 "Leak Channels" To Resting Intracellular Ca2+ In Skeletal Myotubes

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The control of resting free Ca^{2+} ($[Ca^{2+}]_{rest}$) in skeletal muscle is thought to be a balance of channels, pumps (sarcoplasmic reticulum -SR- and plasma membrane ATP-dependent pumps) and exchangers ($Na+/Ca^{2+}$ exchanger). We hypothesized that expression of RyR1 in dyspedic muscle cells, which constitutively lack expression of the skeletal muscle SR Ca^{2+} release, channel, RyR-1, RyR-2, and RyR-3 ($_{Null}$RyR-myotubes) would increase $[Ca^{2+}]_{rest}$ and that this increase would be secondary to passive Ca^{2+} efflux from SR stores mediated by Ry-insensitive leak channels. We explored these mechanisms by measuring $[Ca^{2+}]_{rest}$, using double-barreled Ca^{2+} microelectrodes, in $_{null}$RyR myotubes and myotubes expressing wild-type RyR1 ($_{WT}$RyR1). In addition, changes in $[Ca^{2+}]_{rest}$ produced by several drugs known to modulate the RyR1 channel complex were investigated. We found that $_{WT}$RyR1 myotubes had a 2.0 -fold higher $[Ca^{2+}]_{rest}$ than $_{null}$RyR myotubes. Exposure of both $_{null}$RyR myotubes and $_{null}$RyR myotubes expressing wild-type RyR1 ($_{WT}$RyR1) to 500 μM ryanodine (Ry) or 20 μM (2,6-dichloro-4-aminophenyl) isopropylamine (FLA 365), both of which completely block the caffeine response, had no effects on $[Ca^{2+}]_{rest}$;. However, when $_{WT}$RyR1 myotubes were exposed to a combination of 500 μM Ry and bastadin 5 (B5), $[Ca^{2+}]_{rest}$ was significantly reduced at 23°C in myotubes that express $_{wt}$RyR1 and was reduced to essentially $_{null}$RyR levels at 37°C but had no effect in $_{null}$RyR cells. These results show that expression of RyR1 is responsible for more than half of $[Ca^{2+}]_{rest}$ seen in *Wt* cells and this increase over dyspedic levels is not the result of active gating of the RyR1 channel but instead can be accounted for by RyR1's ryanodine insensitive leak conformation. Supported by NIH PO1 AR052534 (PDA, INP).

1199-Pos Board B43

Prediction of Twitch and High Frequency Local Calcium Dynamics in Mouse EDL Fibers at 15-35°C

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Sites of calcium release, uptake and action are highly organized and densely packed in skeletal muscle cells. This organization suggests an important role for the spatial distribution of organelles and calcium sensitive proteins in muscle function. The current measurement techniques are not able to measure cytosolic [Ca2+] with both high temporal and high spatial resolution. In addition, it is known that all processes are highly temperature dependent. Therefore, the model of Groenendaal et al. [1], that describes local calcium dynamics at physiological frequencies at 35°C, was extended with Q10 correction factors and calcium-fluorescent dye binding kinetics, to predict local calcium dynamics at 15-35°C.

For model validation, simultaneously calcium fluorescent dye and force kinetic measurements were performed. Hereto, murine EDL muscles were isolated and mounted in the set up. Muscle length was adjusted to optimal resting length and stimulation voltage was adjusted to generate maximal force. Rhod-2 AM was loaded in whole muscle at 37°C for 30 minutes or until force was decreased with >25%. Muscles were stimulated at 15-35°C at frequencies between twitch and tetanus.

A significant relation was found between the temperature and the fluorescence characteristics, e.g. decrease in decay time with increasing temperature (one-way ANOVA, p-value<0.05). Model simulations predicted a comparable range. In addition, simulations showed an approximately five-fold difference in calcium gradient throughout the sarcomere between 15 and 35°C.

The validated model is now able 1. to explain calcium fluorescent dye measurements, 2. to translate data at unphysiological to physiological temperature and 3. to predict local calcium dynamics at low and high frequency stimulations at a wide temperature range.

[1] Groenendaal et al. IET Systems Biology, in press.

1200-Pos Board B44

Effects of Changes in Extracellular Concentration of Na^+ and K^+ ($[Na^+]_o$, $[K^+]_o$) on the Ca^{2+} Release Elicited by High Frequency Stimulation. Implications for Muscle Fatigue

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Changes in $[Na^+]_o$ and $[K^+]_o$ occurring during high frequency stimulation has been proposed as a cause of muscle fatigue. We investigated this hypothesis by measuring the Ca^{2+} release elicited by short high frequency trains (100Hz, 10 pulses) in rested frog semitendinosus fibers exposed to various $[Na^+]_o$ or $[K^+]_o$. Myoplasmic $[Ca^{2+}]$ changes (Ca^{2+} transients) elicited by action potentials (AP) were estimated from Ca^{2+}-dependent OGB-5N fluorescence changes. Segments of fibers, stretched to 4.5-5μm, were mounted in an inverted double grease-gap chamber placed in an inverted microscope equipped for epifluorescence. Fibers were held at -100mV and stimulated with 0.5ms current pulses. Normal Ringer solution contained (mM): 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 10 MOPS, 10 dextrose, pH=7.2 with NaOH. $[K^+]_o$ ($[Na^+]_o$) was increased (reduced) by equimolar replacement with Na^+ (N-methyl-D-glucamine). Fibers were loaded (30min) with a solution containing (mM): 110 aspartate, 5 ATP-K_2, 5 Na_2-creatine-phosphate, 20 MOPS, 0.05-0.1 EGTA, 5 $MgCl_2$, 0.2 OGB-5N, pH=7.2 with KOH. A complex interaction of the effects of changing $[K^+]_o$ or $[Na^+]_o$ on membrane potential, AP overshoot and duration, and Ca^{2+} release was found. Using normal Ringer, the amplitude of Ca^{2+} transients elicited by single pulses increased with depolarizations up to -65mV. Raising $[K^+]_o$ had a dual effect on Ca^{2+} release. Ca^{2+} transient's amplitude increased between 2.5 to 10 mM, and decreased markedly for higher $[K]_o$. Potentiation of Ca^{2+} release, but not depression, could be reverted by current injection. This suggests a depolarization independent effect of K^+ ions on Ca^{2+} release.